

# Structural Basis for the Guanosine Requirement of the Hairpin Ribozyme<sup>†</sup>

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*Received August 30, 1999; Revised Manuscript Received September 29, 1999*

**ABSTRACT:** To form a catalytically active complex, the essential nucleotides of the hairpin ribozyme, embedded within the internal loops of the two domains, must interact with one another. Little is known about the nature of these essential interdomain interactions. In the work presented here, we have used recent topographical constraints and other biochemical data in conjunction with molecular modeling (constraint-satisfaction program MC-SYM) to generate testable models of interdomain interactions. Visual analysis of the generated models has revealed a potential interdomain base pair between the conserved guanosine immediately downstream of the reactive phosphodiester ( $G_{+1}$ ) and  $C_{25}$  within the large domain. We have tested this former model through activity assays, using all 16 combinations of bases at positions +1 and 25. When the standard ribozyme was used, catalytic activity was severely suppressed with substrates containing  $U_{+1}$ ,  $C_{+1}$ , or  $A_{+1}$ . Similarly, mutations of the putative pairing partner ( $C_{25}$  to  $A_{25}$  or  $G_{25}$ ) reduce activity by several orders of magnitude. The  $U_{25}$  substitution retains a significant level of activity, consistent with the possible formation of a G·U wobble pair. Strikingly, when combinations of Watson–Crick (or wobble) base pairs were introduced in these positions, catalytic activity was restored, strongly suggesting the existence of the proposed interaction. These results provide a structural basis for the guanosine requirement of this ribozyme and indicate that the hairpin ribozyme can now be engineered to cleave a wider range of RNA sequences.

The hairpin ribozyme is a small catalytic RNA motif isolated from the minus strand of the tobacco ringspot virus satellite RNA. It acts as an endonuclease that catalyzes a reversible sequence-specific cleavage reaction within a substrate RNA (1). The hairpin ribozyme–substrate complex contains two independently folding domains (2). Several studies indicate that tertiary interactions between conserved segments of the two domains are required to form a catalytically active complex (2–6).

There is no X-ray crystal or complete NMR structure for the hairpin ribozyme (7, 8), and until recently, very few interdomain constraints have been available to guide modeling efforts. Recent cross-links obtained by disulfide linkages (9) and topographical constraints obtained in our laboratory using hydroxyl radical footprinting and photoaffinity cross-linking (10, 11) have provided important information concerning the alignment of the two domains in the docked state. However, little is known about the key structural elements involved in the tertiary interactions between the two domains. The guanosine ( $G_{+1}$ ) immediately downstream of the cleavage/ligation site represents a potential candidate for an interdomain contact, since the RNA cleavage and ligation

reactions catalyzed by the hairpin ribozyme exhibit a strong requirement for this nucleotide (12). Base substitutions at this site reduce catalytic activity by several orders of magnitude, and inhibit the formation of a stable tertiary complex between the two domains of the ribozyme–substrate complex (12, 13). NMR analysis of the isolated substrate-binding domain shows a sheared  $G_{+1}\cdot A_9$  base pair, while the Watson–Crick face of  $G_{+1}$  is unoccupied (7). Finally,  $G_{+1}$  and surrounding nucleotides at the cleavage/ligation site are protected from hydroxyl radical cleavage upon docking of the two domains, with concomitant protection of nucleotides 25–27 within the large domain, and the same regions of the complex have been photochemically cross-linked (10, 11).

In the work presented here, we have used three-dimensional computer modeling using MC-SYM, compensatory base substitutions, and FRET<sup>1</sup> to identify and investigate a potential Watson–Crick base pair between  $G_{+1}$  and the cytosine at position 25. This methodology is one of the few examples of the application of RNA modeling methods based on constraints derived from biochemical data in correctly modeling and then experimentally demonstrating a specific RNA tertiary interaction.

## EXPERIMENTAL PROCEDURES

*Preparation of RNA Oligonucleotides.* RNA was synthesized using solid-phase phosphoramidite chemistry and

<sup>†</sup> This work was supported by grants from the National Institutes of Health (AI 44186) to J.M.B. and from the Medical Research Council of Canada (MT 14504) to F.M. R.P. was supported by a postdoctoral fellowship from the Medical Research Council of Canada.

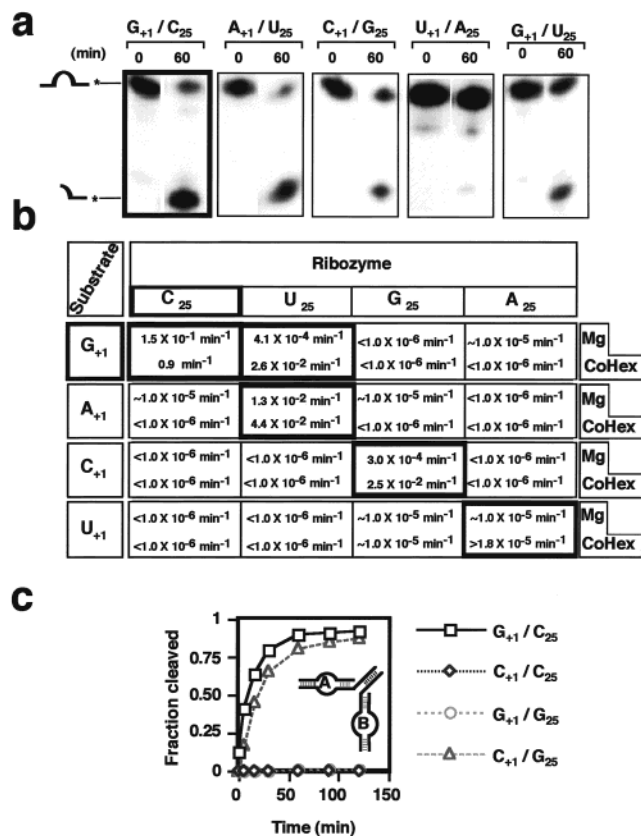
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<sup>1</sup> Abbreviation: FRET, fluorescence resonance energy transfer.

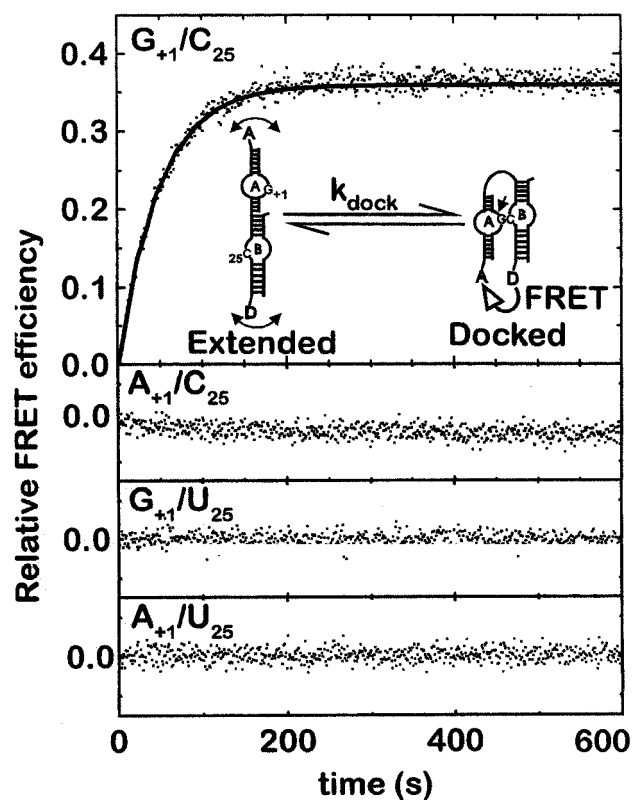




**FIGURE 2:** Cleavage activity of variant ribozymes representing single-base changes at positions 25. (a) Example of restoration of cleavage activity obtained in the presence of 1 mM  $\text{Co}(\text{NH}_3)_6\text{Cl}_3$ . The assembled ribozymes were assayed for cleavage activity against 5'-labeled substrates with or without a substitution at the +1 position. Full-length and cleaved substrates are indicated on the left. The variant combinations and the time points are indicated on top of each autoradiograph. The bold rectangle indicates the wild-type combination. (b) Cleavage rates obtained for the 16 combinations of bases at positions +1 and 25. The first row of each combination indicates the first-order cleavage rate observed in the presence of 12 mM  $\text{MgCl}_2$  and the second row in the presence of 1 mM  $\text{Co}(\text{NH}_3)_6\text{Cl}_3$ . The combinations allowing significant cleavage activity are indicated by bold rectangles. (c) Example of the cleavage activity restoration that can be achieved when reaction conditions [1 mM  $\text{Co}(\text{NH}_3)_6\text{Cl}_3$ ] and RNA constructs more highly favorable for alignment and docking of the two domains are used. In the construct that is shown, the normal hinge region between the two domains is replaced by six unpaired cytidines and a seven-base pair helix (J. E. Heckman and J. M. Burke, unpublished observations).

affect the substrate binding (19). Here, the same cleavage rates were observed when different ribozyme concentrations (100, 250, and 500 nM) were used, indicating that the rate measurements were carried out at saturating conditions for each of the active variants (data not shown).

Replacement of  $\text{Mg}^{2+}$  with  $\text{Co}(\text{NH}_3)_6^{3+}$  generally enhances activity, particularly in the more severely inhibited variants (Figure 2B). Strikingly, almost full restoration of activity was achieved for  $\text{C}_{+1}\cdot\text{G}_{25}$  when reaction conditions [ $\text{Co}(\text{NH}_3)_6^{3+}$ ] and an RNA construct favoring proper alignment of the two domains were used (Figure 2C). These results establish that a base pair between positions +1 and 25 is required for catalytic function of the hairpin ribozyme. A structural model consistent with these observations is presented below. Using analogous combinations of base sub-



**FIGURE 3:** Global folding transition from an extended to a docked complex. Docking of the two domains is accompanied by an increase in the rate of fluorescence resonance energy transfer (FRET) between a domain-terminal donor–acceptor fluorophore pair (13). While the wild-type construct ( $\text{G}_{+1}\cdot\text{C}_{25}$ ) docks to a high extent, none of the tested single or double mutations leads to a detectable docked conformation at up to 100 mM  $\text{MgCl}_2$  or 1 mM  $\text{Co}(\text{NH}_3)_6\text{Cl}_3$ , or a combination of the two (see Experimental Procedures for details). Only three variants are shown here. Similar results were obtained with other active or inactive combinations.

stitutions, we were unable to demonstrate covariation between position +1 or +2 in the substrate and position 24 or 26 of the ribozyme catalytic domain (data not shown).

Following substrate binding, the two domains interact with one another forming a docked complex, as previously shown by FRET (13) and footprinting (10) methods. The rate-limiting step of cleavage follows this docking event (13). Although the identity of the rate-limiting step is unknown, its pH independence suggests that it may be a post-docking conformational change, rather than reaction chemistry (13, 20). Some conditions (e.g., high concentrations of monovalent cations) strongly support cleavage, yet do not favor the formation of a stable docked complex (21). Analysis of the variants used in this study gave similar results, in that although activity was rescued, the stability of the docked complex is diminished in all active +1 and 25 variants, resulting in a population of docked molecules that is below the limit of detection of the FRET assay (Figure 3). Together, these results indicate that, following a transient docking step, the active variants can proceed to the transition state conformation leading to cleavage.

The interdomain base pair between positions +1 and 25 clearly serves as a key structural determinant of the reactive complex. MC-SYM models of the standard complex suggest that the  $\text{G}_{+1}\cdot\text{A}_9$  sheared pair found within the ground state

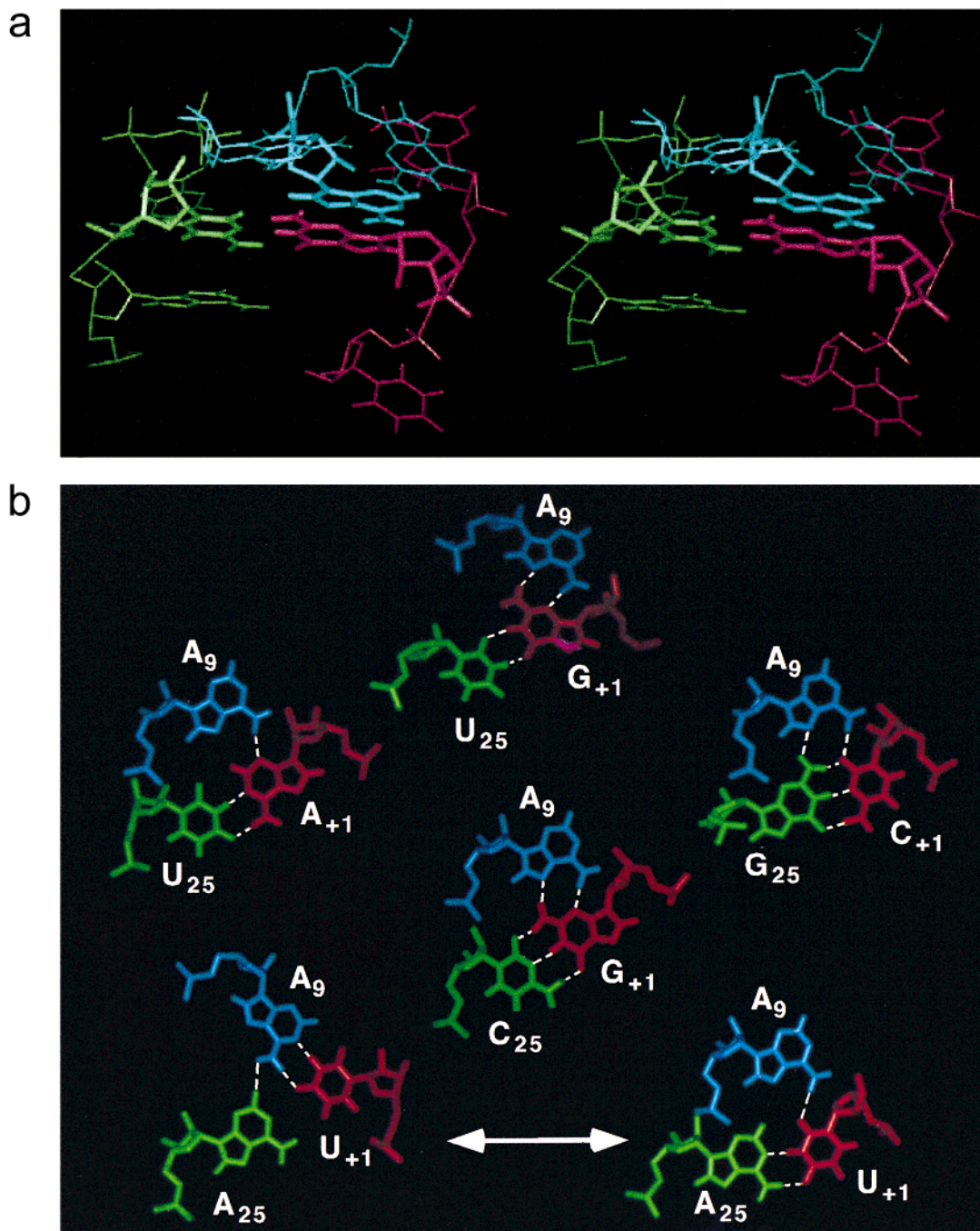


FIGURE 4: Molecular model of the  $A_9 \cdot G_{+1} \cdot C_{25}$  triplet. (a) Stereoview showing the proposed base triple interaction involving  $G_{+1}$  (red) and its partners  $A_9$  (blue) and  $C_{25}$  (green). The nucleotides immediately 5' and 3' of the triplet also are shown (thinner lines). (b) Secondary structure of the proposed base triple. Compensatory variants of the triplet analyzed in this work are also indicated. The potential formation of a Watson–Crick base pair between  $U_{+1}$  and  $A_9$  in the internal loop A may impair triplet formation and is consistent with the low level of restoration of cleavage activity of that combination (bottom).

structure of internal loop A (7) can readily accommodate formation of the  $G_{+1} \cdot C_{25}$  pair, which could lead to the formation of an  $A_9 \cdot G_{+1} \cdot C_{25}$  triplet (Figure 4a). This appealing hypothesis is currently under investigation in our laboratory. Interestingly, analogous triplets can be formed by the other active combinations, and the activities of the variants appear to correlate with their ability to adopt orthologous structures (Figure 4b). The very low activity observed for the  $U_{+1} \cdot A_{25}$  pair is consistent not only with the formation of a weak triplet but also with possible formation of a  $U_{+1} \cdot A_9$  pair that could disrupt the interdomain contact (Figure 4b).

The  $G_{+1} \cdot C_{25}$  base pair represents a useful starting point from which to build a tertiary structure model of the ribozyme–substrate complex. The proven interdomain contact between  $G_{+1}$  and  $C_{25}$  is also significant for targeted RNA cleavage by hairpin ribozymes, since targets are no longer limited to sequences containing a guanosine immediately 3' to the cleavage site.

#### ACKNOWLEDGMENT

We dedicate this paper to the memory of Robert Cedergren. We thank Ken Hampel, Molly Coseno, Sébastien

Lemieux, Patrick Gendron, and Nancy Bourassa for valuable comments and helpful discussions about the manuscript.

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BI992024S